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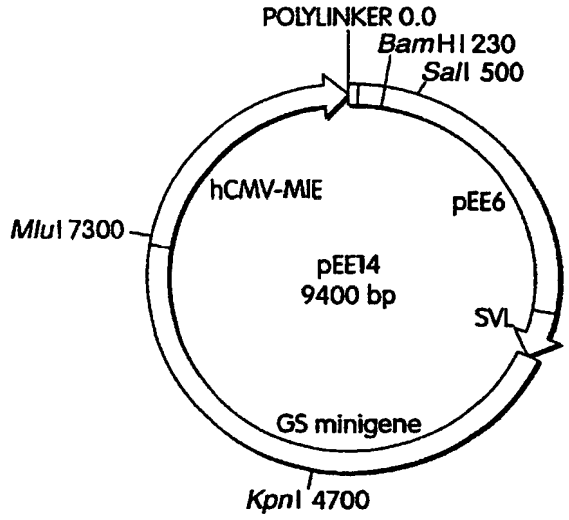

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(57) Abstract

The invention features a nucleic acid, vector, cells, and processes for expressing recombinant human alpha-fetoprotein (rHuAFP) in cultured mammalian cells.

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EXPRESSION OF HUMAN ALPHA-FETOPROTEIN IN MAMMALIAN CELLS

Background of the Invention

This invention relates to the production of recombinant protein expressed in mammalian cells.

Alpha-fetoprotein (AFP) is a 70 kDa glycoprotein produced by the yolk sac and fetal liver. AFP is present in fetal serum at milligram levels, and, at birth, declines to the nanogram levels normally found in adult serum. Increased levels of AFP in adult serum are indicative of a yolk sac tumor, a hepatoma, or of liver regeneration. The role of AFP during fetal development is not known, although it has been suggested that AFP may protect a gestating fetus from a maternal immune attack or from the effects of maternal estrogen.

In vitro and *in vivo* experiments have shown that AFP has both cell growth-stimulatory and -inhibitory activities, depending upon the target cell, the relative concentration of AFP, and the presence of other cytokines and growth factors. For example, AFP can inhibit the growth of many types of tumor cells, and, in particular, inhibits estrogen-stimulated cell growth. Conversely, AFP stimulates the growth of normal embryonal fibroblasts. AFP has also been shown to have both immunosuppressive and immunoproliferative effects. In order to exploit the various biological properties of AFP, it will be necessary to obtain sufficient quantities of this molecule in an efficient and cost-effective manner.

Summary of the Invention

In a first aspect, the invention features a substantially pure nucleic acid molecule including: (i) a nucleic acid sequence encoding recombinant human alpha-fetoprotein (rHuAFP), (ii) a promoter that drives transcription in a mammalian cell, wherein the promoter is operably linked to the rHuAFP-encoding sequence, wherein the promoter directs expression of the rHuAFP in a mammalian cell, and (iii) a heterologous leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by the mammalian cell.

In a second aspect, the invention features a vector including the substantially pure nucleic acid molecule of the first aspect of the invention.

In a third aspect, the invention features a mammalian cell including the substantially pure nucleic acid molecule of the first aspect of the invention.

In various embodiments of the third aspect of the invention, the substantially pure nucleic acid molecule may be stably integrated into the genome of the mammalian cell or the substantially pure nucleic acid molecule may be contained within a vector that is episomally maintained by the mammalian cell.

In a fourth aspect, the invention features a method of producing recombinant human alpha-fetoprotein (rHuAFP) in a mammalian cell *in vitro*. The method includes: (a) providing a mammalian cell containing a DNA expression construct that includes: (i) a nucleic acid sequence encoding rHuAFP and (ii) a promoter that drives transcription in a mammalian cell, wherein the promoter is operably linked to the rHuAFP-encoding sequence, wherein the promoter directs expression of the rHuAFP in a mammalian cell; (b) culturing the mammalian cell such that the mammalian cell expresses rHuAFP; and (c) purifying rHuAFP produced by the mammalian cell.

In one embodiment of the fourth aspect of the invention, the rHuAFP may be obtained from a lysate of the mammalian cell. In another embodiment of the fourth aspect, the DNA expression construct may further comprise a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by the mammalian cell, and rHuAFP is obtained from the cell culture medium conditioned by the cell. In yet another embodiment of the fourth aspect, the leader sequence may be a heterologous leader sequence.

In still other embodiments of the fourth aspect, the DNA expression construct may be stably integrated into the genomic DNA of the mammalian cell, the DNA expression construct may be carried within a vector that is episomally maintained by the mammalian cell, or the DNA expression construct may be carried within a vector that is transiently transfected into the cell.

By "human alpha-fetoprotein" or "HuAFP" is meant a polypeptide having substantially the same amino acid sequence as the mature alpha-fetoprotein (amino acids 20-609) set forth in Genbank Accession No. J00077, encoded by the cDNA sequence set forth in Genbank Accession No. J00077, and reported in Morinaga et al. (*Proc. Natl. Acad. Sci.* USA 80:4604-4608, 1983).

By "human alpha-fetoprotein precursor" is meant a polypeptide having substantially the same amino acid sequence as amino acids 1-609 set forth in Genbank Accession No. J00077, encoded by the cDNA sequence set forth in Genbank Accession No. J00077, and reported in Morinaga et al. (*Proc. Natl. Acad. Sci.* USA 80:4604-4608, 1983).

By "human alpha-fetoprotein secretory signal" or "human alpha-fetoprotein signal peptide" is meant amino acids 1-19 set forth in Genbank Accession No. J00077. The protein secretory signal is cleaved from HuAFP

during the process of protein maturation and secretion.

By "substantially identical" is meant a polypeptide that exhibits at least 80% homology with a naturally-occurring HuAFP amino acid sequence, typically at least 85% homology with a naturally-occurring human HuAFP sequence, more typically at least 90% homology, usually at least 95% homology, and most often at least 97% homology with a naturally-occurring HuAFP sequence. The length of comparison sequences will generally be at least 16 amino acids, usually at least 20 amino acids, preferably at least 25 amino acids, more preferably at least 30 amino acids, and most preferably more than 35 amino acids.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those transcriptional control elements (e.g., enhancers, repressors, and silencers) that are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "recombinant HuAFP" or "rHuAFP" is meant human alpha-fetoprotein encoded by a substantially pure HuAFP-encoding nucleic acid isolated by techniques such as DNA cloning, polymerase chain reaction (PCR) amplification, chemical synthesis, or any other known method by which an rHuAFP-encoding nucleic acid may be obtained.

By "exogenous" is meant a nucleic acid that is introduced by artifice into a cell, or a polypeptide encoded by such a nucleic acid.

By "purified" is meant that rHuAFP is partially or completely separated from other components (e.g., proteins, lipids, carbohydrates, nucleic acid, salts, and water) present in cells or cell culture medium, thus increasing the effective concentration of rHuAFP relative to unpurified rHuAFP present in cells or cell culture medium.

By "substantially pure nucleic acid" is meant nucleic acid that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into: a vector, an autonomously replicating plasmid or virus, or the genomic DNA of a prokaryote or eukaryote, or that exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene containing a nucleotide sequence not native to the gene or encoding additional polypeptide sequence, as well as the corresponding mRNA.

By "transformation" or "transfection" or "transduction" is meant any method for introducing foreign DNA into a cell. Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, transduction (e.g., bacteriophage, adenoviral retroviral, or other viral delivery), electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

By "transformed cell" or "transfected cell," or "transduced cell," is meant a cell (or a descendent of a cell) into which a DNA molecule encoding rHuAFP has been introduced, by means of recombinant DNA techniques. The

DNA molecule may be stably incorporated into the host chromosome or may be episomally maintained.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "expression vector" is meant a genetically engineered plasmid or virus, derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, herpesvirus, vaccinia virus, Semliki Forest Virus, or artificial chromosome, that is used to transfer an rHuAFP coding sequence, operably linked to a promoter and any other necessary sequences (e.g., polyadenylation and splice sites, introns, enhancers, and signal sequences), into a host cell, such that the encoded rHuAFP is expressed within the host cell.

By "expression construct" is meant an rHuAFP coding sequence operably linked to a promoter and any other sequences necessary for expression of rHuAFP within a host cell. The expression construct may be carried within a plasmid or viral expression vector.

By "leader sequence" or "signal sequence" is meant a nucleic acid sequence that encodes a protein secretory signal, that, when operably linked to a downstream nucleic acid molecule encoding rHuAFP, directs rHuAFP secretion. The leader sequence may be the native rHuAFP leader (which encodes amino acids 1-19 set forth in Genbank Accession No. J00077), an artificially-derived leader, a leader from the same gene as the promoter used to direct transcription of the rHuAFP coding sequence, or a leader from any other protein that is normally secreted from a cell.

By "culture medium" is meant any aqueous solution that is used to maintain living cells *in vitro*. One example of a culture medium is Glasgow-modified Eagle medium. Many other examples of culture media are known to skilled artisans.

By "conditioned medium" is meant culture medium into which cells have secreted rHuAFP.

By "heterologous" is meant an amino acid or nucleic acid sequence that is not a HuAFP (or HuAFP precursor) amino acid or nucleic acid sequence.

By "stably integrated" is meant any piece of nucleic acid (e.g., an expression construct) that is inserted by artifice into a cell and becomes physically incorporated into the chromosomal DNA of that cell, such that the inserted nucleic acid is transmitted along with the chromosomal DNA to daughter cells resulting from each successive cell division. The nucleic acid may encode a gene product that is foreign to the cell into which the nucleic acid is introduced (e.g., an rHuAFP-encoding nucleic acid introduced into a mouse cell) or a gene product that is encoded by an endogenous gene within the cell (e.g., an rHuAFP-encoding nucleic acid introduced into a human cell).

By "episomally carried" is meant a that an expression vector artificially introduced into a cell may be maintained within the cell and passed along to successive generations of daughter cells without having physically integrated into the chromosomal DNA of the ancestor cell. An episomally carried vector may be, e.g., an artificial chromosome or an expression vector that contains an origin of replication that functions in mammalian cells, such as the Epstein-Barr Virus oriP (which functions in mammalian cells expressing EBNA-1) or the SV40 origin of replication (which functions in mammalian cells expressing SV40 large T antigen).

Brief Description of the Drawing

Fig. 1 is a diagram of the pEE14 GS expression vector.

Fig. 2(A-B) is a representation of DNA fragments that encode rHuAFP.

Detailed Description of the Invention

The present invention features a process for obtaining purified recombinant human alpha-fetoprotein (rHuAFP) expressed by mammalian cells. A DNA fragment encoding rHuAFP is first cloned into an appropriate mammalian expression vector such that the rHuAFP will be expressed under the transcriptional control of a promoter contained within the vector. The rHuAFP-encoding vector (or a fragment thereof containing the promoter, rHuAFP coding sequences, and other necessary regulatory sequences) is then introduced into an appropriate mammalian cell line. Cells that express rHuAFP may be identified, for example, by growing the rHuAFP-transformed cells in the presence of a selective agent that kills cells not containing an HuAFP expression construct. The surviving cells may subjected to further selective pressure and then cloned to obtain cell lines that express rHuAFP at the desired levels.

In order to be actively secreted, rHuAFP must be preceded by a protein secretory signal. rHuAFP lacking a secretory signal is purified from cell lysates. rHuAFP preceded by a secretory signal may be purified from medium conditioned by rHuAFP-expressing cells.

A detailed description of the various mammalian expression systems and methods for expressing recombinant proteins in mammalian cells are provided in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., New York, NY, 1997, pp. 16.12.1-16.20-16 and A.5.23-

A.5.30).

Expression Constructs

rHuAFP expression constructs contain a promoter together with any other transcriptional regulatory sequences (for example, enhancers and silencers) necessary for expression of rHuAFP in the chosen mammalian cell type. Any promoter or enhancer that drives transcription of an operably linked gene in a mammalian cell may be used to express rHuAFP. A promoter or enhancer may be from a mammalian gene, such as β-actin or globin, or may be from, e.g., a gene from a virus that infects mammalian cells. Viral promoters and enhancers that are commonly used to efficiently express protein in mammalian cells include, e.g. the cytomegalovirus (CMV) and SV40 early promoters and enhancers. An enhancer may be either upstream or downstream from the rHuAFP coding region. Expression constructs ideally contain a convenient restriction site downstream from the promoter, into which a DNA fragment encoding rHuAFP is cloned such that the promoter is operably linked to the rHuAFP gene.

Expression constructs may include a leader sequence downstream from the promoter. The leader sequence is a nucleic acid segment that encodes a protein secretory signal, and, when operably linked to a downstream nucleic acid molecule encoding rHuAFP, directs rHuAFP secretion. Preferably, the leader sequence (nucleotides 45-101 of Genbank Accession No. J00077) encoding the native rHuAFP protein secretory signal (amino acids 1-19 of Genbank Accession No. J00077) is employed. Other options include use of: a leader sequence that encodes a protein secretory signal from any other protein that is normally secreted from a cell (e.g., a protein hormone such as insulin), an artificial leader sequence that encodes an artificial protein secretory signal,

or a chimeric leader sequence.

If desired, expression of rHuAFP may be regulated by using one of the inducible or repressible promoter systems that are known to skilled artisans. The advantage of a regulatable promoter is that rHuAFP is expressed only during a specified period of time (i.e., immediately prior to purification), thereby minimizing the effect of expressed rHuAFP on the cells during periods of cell maintenance or growth.

One example of an inducible promoter is the metallothionein I promoter, which is transcriptionally silent in the absence of zinc. The addition of zinc to the cell culture medium induces transcription of any gene (such as an rHuAFP gene) that is operably linked to the metallothionein I promoter (see, e.g., Ellouk-Achard et al., *J. Hepatol.*, 5:807-818, 1998; Jorge et al., *Appl. Microbiol. Biotechnol.*, 46:533-537, 1996; and Prostko et al., *Oncol. Res.*, 9:13-17, 1997).

rHuAFP may also be placed under the transcriptional regulation of steroid hormone-inducible promoters, which contain DNA binding sites for hormones such as glucocorticoids. The promoter is inactive in the absence of steroid hormone; when steroid hormone (e.g., dexamethasone) is added to the cell culture medium, promoter activity is induced, and cells containing the promoter-rHuAFP-encoding construct express rHuAFP (see, e.g., Mader and White, *Proc. Nat. Acad. Sci. USA*, 90:5603-5607, 1993; and Hirt et al., *Gene*, 111:199-206, 1992).

Expression vectors containing promoters that are inducible or repressible by tetracycline (or tetracycline derivatives such as doxycycline) in a dose-dependent manner may also be used to express rHuAFP in mammalian cells. For example, pRetro-On (Clontech, Palo Alto, CA; Catalog # 6157-1) is a retroviral vector that contains a tetracyline-induced promoter. In the presence

of tetracycline, a vector-encoded reverse tetracyline-controlled transactivator (rtTA) binds to a tet-responsive element (TRE) and activates transcription in a dose-dependent manner. Conversely, pRetro-Off (Clontech, Palo Alto, CA; Catalog # 6158-1) is a retroviral vector containing a tetracyline-repressed promoter. In the absence of tetracycline, a vector-encoded tetracyline-controlled transactivator (tTA) binds to a tet-responsive element (TRE) and activates transcription. Transcription is repressed in a dose-dependent manner by the addition of tetracycline to the cell culture medium.

In addition to transcriptional regulatory elements, expression constructs for expressing rHuAFP in mammalian cells also preferably include additional elements that regulate mRNA stability and translation into protein. Such elements include, e.g., DNA sequences for 5' and 3' untranslated regions (UTRs), a transcription termination site, a signal for cleavage and polyadenylation of the transcribed mRNA, and a translation termination signal. These elements are all well known in the art.

Expression constructs may further include an intron that increases the level of expression of the encoded rHuAFP. The intron may be placed between the transcription initiation site and the translational start codon, 3' of the translational stop codon, or within the rHuAFP coding region. The intron should include a 5' splice site (i.e., a donor site), a 3' splice site (i.e., an acceptor site), and preferably, at least 100 nucleotides between the two sites. Any intron that is known in the art to increase expression of an exogenously-introduced gene (e.g., the SV40 small t intron or a chimeric intron, e.g., the intron in pED (Kaufman et al., *Nuc. Acids Res.* 19:4485-4490, 1991), which consists of the 5' splice site from the first leader of the adenovirus major late mRNAs and a 3' splice site from an immunoglobulin gene) or the intron in pCI-neo (Promega, Madison, WI; Catalog # E1841), which consists of the 5' splice site from the β-

globin intron and the 3' splice site from an IgG intron, may be used.

The rHuAFP expression construct may be carried within a circular plasmid or other vector, such as a vector derived from a virus. The vector may contain additional sequences that facilitate its propagation in prokaryotic and eukaryotic cells, for example, drug-selectable markers (e.g., for ampicillin resistance in *E. coli*, or G-418, puromycin, methotrexate, or methionine sulfoxamine resistance in mammalian cells) and origins of replication (e.g., colE1 for replication in prokaryotic cells, and oriP from Epstein-Barr virus for replication in mammalian cells that express EBNA-1, or the SV40 origin of replication for cells that express SV40 large T antigen).

Examples of viral and plasmid expression vectors for expressing proteins in cultured mammalian cells are provided in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., New York, NY, 1997, pp. 16.12.1-16.20-16 and A.5.23-A.5.30).

Introduction of Expression Vectors into Cells

rHuAFP expression constructs may introduced into target cells by transfection, electroporation, transduction, or microinjection; numerous methods for performing these techniques are well known to skilled artisans. When highly efficient methods such as viral transduction are used, a large percentage of the transduced cells will take up the expression construct. rHuAFP expressed by such cells may be isolated without an intervening selection step. However, in most cases, it will be desirable to subject the cells to at least one selection step in order to isolate cells that contain the expression construct.

The selection is done by exposing the cells to a compound that kills or inhibits the growth of cells not expressing a marker gene encoded by the expression construct or vector, or a co-introduced vector. Generally, the selection yields cells that have stably integrated the expression construct into their genomic DNA. Cells that contain high copy numbers of a rHuAFP expression construct may be obtained by performing an amplification step, in which the cells are placed under even greater selective pressure by increasing the concentration of the selective agent. Cells emerging from the selection may then be cloned using cloning cylinders or by limiting dilution. rHuAFP expression levels in the clonal cell lines may then be determined using methods such as immunoblotting, ELISA, Northern hybridization, RNA dot-blotting, or reverse transcription-PCR (RT-PCR).

Cells

Any mammalian cell line in which an rHuAFP expression construct can be expressed may be used to generate rHuAFP-expressing cell lines. The cell line selected will depend upon various factors, such as the characteristics of the expression vector to be used, and the method by which the vector is to be introduced into the cell.

For example, in order to obtain maximal rHuAFP levels from an rHuAFP expression vector having an SV40 origin of replication (e.g., CDM8), the host cell preferably expresses SV40 large T antigen (e.g., COS-7 cells), because this protein drives high copy replication of vectors containing an SV40 origin of replication (Ausubel et al., *supra*).

The selectable marker chosen for generation of rHuAFP-expressing cells also influences selection of the type of host cell used. For example, maximal rHuAFP gene amplification using DHFR as a selectable marker is

most readily achieved using DHFR-minus cell lines such as CHO-K1 (Cockett et al., *Bio/Technology* 8:662-667, 1990).

In choosing an appropriate cell line for maximal rHuAFP production, other factors to consider are ease of transfection/transduction, genetic stability, and ability to grow and express rHuAFP under the culture conditions to be used. For example, use of cells that may be grown or readily adapted to being grown in suspension culture may facilitate large-scale production of rHuAFP (Cockett, *supra*).

Purification of AFP from Cell Lysate or Cell Culture Medium

rHuAFP may be purified from cell lysates or from medium conditioned by rHuAFP-expressing cells using standard protein purification techniques, such as affinity chromatography (see, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998) or other methods known to those skilled in the art of protein purification. Once isolated, the recombinant protein may, if desired, be further purified by *e.g.*, by high performance liquid chromatography (HPLC; e.g., see Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, Eds. Work and Burdon, Elsevier, 1980). Preferably, the purification is by at least 50-fold, more preferably, by at least 100-fold, still more preferably, by at least 500-fold, and yet more preferably, by at least 1000-fold, and most preferably, by at least 10,000-fold.

Use of rHuAFP Purified from Cell Lysate or Conditioned Cell Culture Medium

rHuAFP purified from cell lysate or cell culture medium may be used as a diagnostic standard (e.g., for detection of increased levels of AFP in adult human serum, which may indicate the presence of cancer or liver

regeneration) or as a therapeutic. For example, rHuAFP produced by the methods of the invention may be administered to mammals to inhibit cancer cell growth, to induce bone marrow cell proliferation (for example, after a bone marrow transplant or after administration of a myelotoxic treatment such as chemotherapy), or as an immunosuppressive agent (for example, to treat systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, myasthenia gravis, insulin-dependent diabetes mellitus, or to inhibit rejection of a transplanted organ).

rHuAFP purified from cell lysate or cell culture medium may be administered in an effective amount either alone or in combination with a pharmaceutically acceptable carrier or diluent, either alone or in combination with other therapeutic agents by any convenient means known to skilled artisans, e.g., intravenously, orally, intramuscularly, or intranasally.

In general, rHuAFP produced by the methods described herein may be used as described in Murgita et al. (U.S.P.N. 5,384,250).

Example I: Expression and purification of non-secreted rHuAFP

Cloning an HuAFP-encoding DNA fragment lacking a signal sequence into an expression vector

A DNA fragment encoding full-length HuAFP is cloned into the expression vector pEE14 (Fig. 1). pEE14 (Celltech, Slough, U.K; Cockett et al., *Bio/Technology* 8:662-667, 1990) is an expression vector containing a glutamine synthetase (GS) minigene, driven by an SV40 late promoter, as the selectable marker for the generation of stable cell lines. Vector pEE14 is digested with both EcoRI and BamHI restriction endonucleases and the resulting digested vector is isolated from 0.6% agarose gel using Geneclean (Bio 101, Vista, CA) according to the manufacturer's instructions.

A 1789 base pair (bp) rHuAFP-encoding DNA fragment having an EcoRI site at the 5' end and a BamHI at the 3' end (Fig. 2B; constructed as described in Murgita et al., U.S.P.N. 5,384,250) is recovered from pHuAFP using Geneclean (described in Murgita et al., *supra*), following digestion of the plasmid with EcoRI and BamHI restriction endonucleases. The rHuAFP coding sequence is inserted into the digested pEE14 vector in the direction of EcoRI and BamHI and under the control of the hCMV-MIE promoter-enhancer. Plasmid minipreps from DH5-α transformants are screened by restriction analysis for a vector containing the rHuAFP insert. Positive clones that contain the rHuAFP coding sequence are identified and plasmid DNA is isolated using the QIAprep Miniprep kit (QIAGEN; Los Angeles, CA) and stored at -20°C.

Generation of stably transfected CHO-K1 cells that express HuAFP

CHO-K1 cells (ATCC #CCL61) are maintained in complete Glasgow- modified Eagle medium containing 10% FCS (GMEM-10) (Gibco BRL, Gaithersburg, MA). The day before transfection, the cells are trypsinized and seeded into 100 mm petri dishes at approximately 10^6 cells per dish. The cells are transfected with 10 µg of rHuAFP-encoding plasmid per dish, using calcium phosphate-DNA precipitates formed in BES (N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid. Briefly, the plasmid is mixed with 500 µl of 0.25M $CaCl_2$. Five hundred µl of 2X BES is added, mixed and incubated 10-20 min at room temperature. The calcium phosphate-DNA solution is added dropwise onto the medium-containing plate. The plates are then placed in a 35°C, 3% CO_2 incubator for 24 hours.

After 24 hours, the medium is replaced with selective medium consisting of fresh complete GMEM-10 containing L-methionine sulfoximine (MSX; Sigma, St. Louis, MO) at a final concentration of 25 μM, which is used to select for cells containing rHuAFP expression plasmids. After 4-5 days, the selective medium is replenished. This process is repeated for the next 2 weeks and until the appearance of MSX-resistant colonies. MSX-resistant colonies are isolating using cloning cylinders, and each colony is seeded into petri dishes containing fresh selective medium with various concentrations of MSX, ranging between 100 μm and 1mM. The dishes are incubated 10-14 days during which the medium is changed only once. After this period, the colonies surviving at the highest concentrations of MSX are isolated and expanded, with each colony in a separate petri dish. Once the cells have reached confluency, they are assayed by immunoblot analysis for the presence of rHuAFP. Those cell lines producing the highest levels of rHuAFP are selected for large-scale rHuAFP production.

Expression and purification of rHuAFP from stably transfected CHO-K1 cells

Stably transfected CHO-K1 cells are grown in GMEM-10 containing MSX. Cells are seeded into 150 cm^2 tissue flasks at a density of 10^6 cells per flask and are grown to 100% confluency before harvesting. Medium is removed from the flask, cells are washed 2X in PBS and then subjected to lysis with 5 ml of lysis buffer (25 mM Tris-HCI, pH 7.6, 0.15 M NaCl, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The resulting suspensions are removed from the flasks and gently sonicated. The lysate is cleared by centrifugation at 7500 x g for 15 min and then dialyzed against 20 mM Tris-HCl, pH 8.0. Final purification of rHuAFP is achieved by applying the dialyzed sample onto a Mono Q column

equilibrated in 20 mM Tris-HCl, pH 8.0. Bound proteins are eluted during a step gradient from 0-100% (1 M NaCl, 20 mM Tris-HCl, pH 8.0). Fractions containing rHuAFP are identified using ELISA or immunoblotting.

Example II: Expression and purification of secreted rHuAFP

Cloning an HuAFP fragment containing a signal sequence into an expression vector

The coding sequence for full-length AFP and its signal sequence are cloned into the expression vector pEE14 (Celltech) (Fig. 1). pEE14 is prepared as described in Example I. A DNA fragment encoding the rHuAFP precursor protein (rHuAFP preceded by a signal peptide) is generated using plasmid pLHuAFP (Murgita, *supra*; Fig. 1A) as a template and the following oligonucleotide primers: NH_2 (5'-AAA GAA TTC ATG AAG TGG GTG GAA-3') and COOH (5-AAA GGA TCC TTA AAC TCC CAA AGC-3') to PCR-amplify a DNA segment encoding the HuAFP precursor, which contains a secretory signal. Each PCR reaction contains 34 µl H_2O, 10 µl 10X reaction buffer, 20 µl 1mM dNTPs, 2 µl DNA template, 10 µl of 10 pmol/µl 5'-primer, 10 µl of 10 pmol/µl 3'-primer, 1 µl glycerol, 10 µl DMSO and 1 µl *Pfu* DNA polymerase. Annealing, extension, and denaturation temperatures are 50°C, 72°C, and 94°C, respectively, for 30 cycles, using the Gene Amp PCR System 9600 (Perkin-Elmer, Norwalk, CN). The 1840 bp DNA fragment obtained from the PCR reactions is purified by isolating the fragment from a 0.7% TAE agarose gel, followed by gel extraction using Geneclean. The PCR amplification product is then digested with EcoRI and BamHI and inserted into the vector pEE14 as described in Example I. Stably transfected clones expressing secreted rHuAFP are obtained as described in Example I.

Purification of secreted rHuAFP from medium conditioned by stably transfected CHO-K1 cells

Stable transfections of CHO-K1 cells are grown in GMEM-10 containing the appropriate concentration of MSX (determined as described in Example I). Cells are seeded in 150 cm^2 tissue flasks at a density of 10^6 cells per flask and are grown to 100% confluency before harvesting the conditioned medium. Medium is removed from the flask and cleared of cells by centrifugation at 1000 x g for 15 min. The rHuAFP-containing medium is concentrated 10-20 fold by ultrafiltration with a YM30 Amicon membrane (Millipore, Bedford, MA) and dialyzed overnight against 20 mM Tris-HCl, pH 8.0. Final purification of rHuAFP is achieved by applying the dialyzed sample onto a Mono Q column equilibrated in 20 mM Tris-HCl, pH 8.0. Bound proteins are eluted during a step gradient from 0-100% (1 M NaCl, 20 mM Tris-HCl, pH 8.0). Fractions containing rHuAFP are identified using ELISA or immunoblotting.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention

pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

1. A substantially pure nucleic acid molecule comprising: (i) a nucleic acid sequence encoding recombinant human alpha-fetoprotein (rHuAFP), (ii) a promoter that drives transcription in a mammalian cell, wherein said promoter is operably linked to the rHuAFP-encoding sequence to direct expression of said rHuAFP in a mammalian cell, and (iii) a heterologous leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by said mammalian cell.

2. A vector comprising the substantially pure nucleic acid molecule of claim 1.

3. A mammalian cell comprising the substantially pure nucleic acid molecule of claim 1.

4. The mammalian cell of claim 3, said wherein said substantially pure nucleic acid molecule is stably integrated into the genome of said mammalian cell.

5. The mammalian cell of claim 3 said wherein said substantially pure nucleic acid molecule is contained within a vector that is episomally maintained by said mammalian cell.

6. A method of producing recombinant human alpha-fetoprotein (rHuAFP), said method comprising:

(a) providing a mammalian cell containing a DNA expression construct that comprises: (i) a nucleic acid sequence encoding r HuAFP and (ii) a promoter that drives transcription in a mammalian cell, wherein said promoter is operably linked to the rHuAFP-encoding sequence to direct expression of said rHuAFP in a mammalian cell;

(b) culturing said mammalian cell such that said mammalian cell expresses rHuAFP; and

(c) purifying rHuAFP produced by said mammalian cell.

7. The method of claim 6, wherein said DNA expression construct further comprises a leader sequence encoding a protein secretory signal that enables secretion of said rHuAFP by said mammalian cell, and wherein said rHuAFP is obtained from medium conditioned by said mammalian cell.

8. The method of claim 7, wherein said leader sequence is a heterologous leader sequence.

9. The method of claim 6, wherein said DNA expression construct is stably integrated into the genomic DNA of said mammalian cell.

10. The method of claim 6, wherein said DNA expression construct is carried within a vector that is episomally maintained by said mammalian cell.

11. The method of claim 6, wherein said DNA expression construct is transiently transfected into said cell.

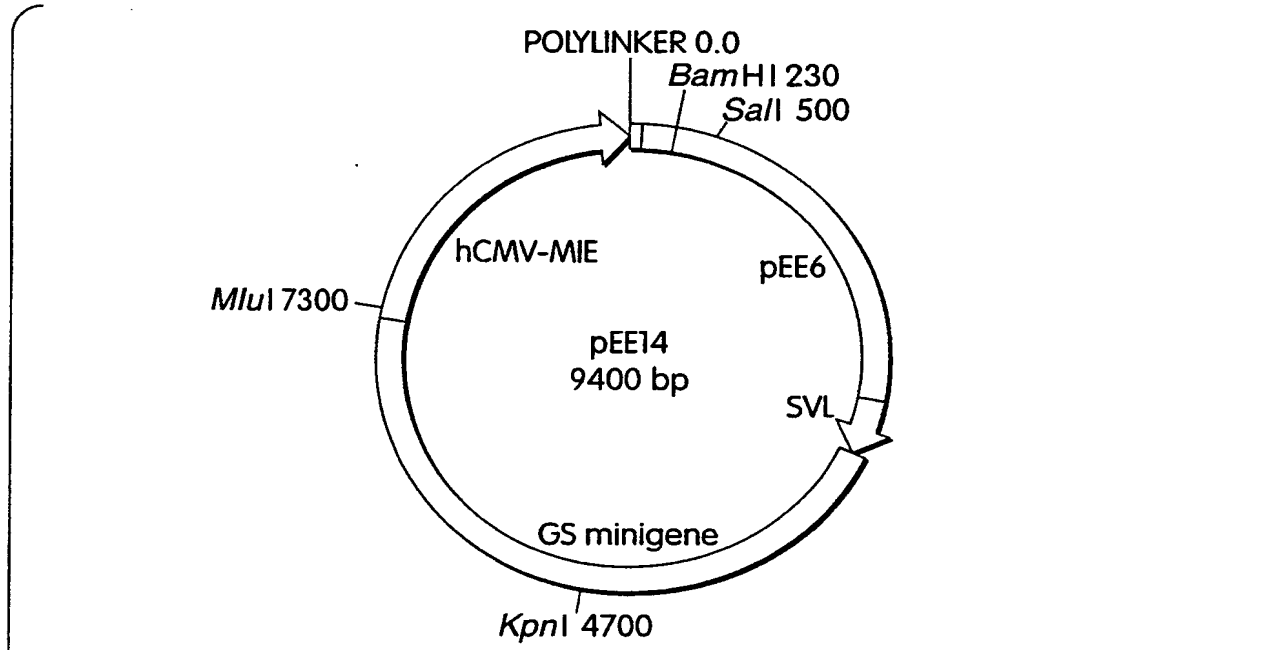

Fig. 1

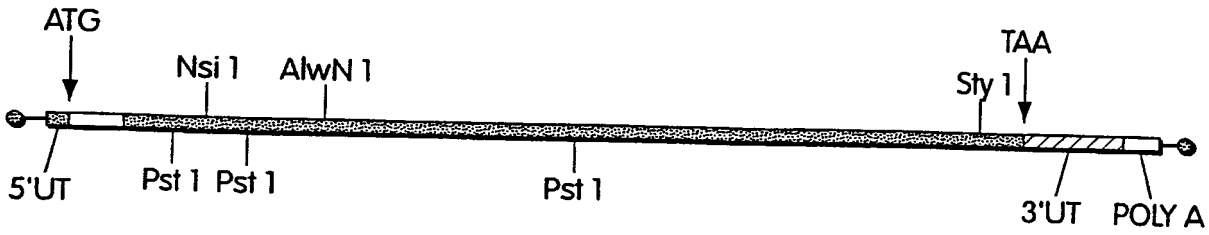

Fig. 2A

Fig. 2B

INTERNATIONAL SEARCH REPORT

Internat[redacted]pplication No.
PCT/US00/06038

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/12; A61K 37/02, 37/04, 35/12
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/8, 2; 530/387.1, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CATURLA et al. The Thyroid hormone down-regulates the mouse alpha-feotoprotein promoter. Molecular and Cellular Endocrinology. December 1997, Vol. 135, No. 2, pages 139-145, see entire document.	1-11
Y	GALARNEAU et al. The alpha-1-Fetoprotein Locus is Activated by a Nuclear Receptor of the Drosophila FTZ-F1 Family. Molecular and Cellular Biology. July 1996, Vol. 16, No. 7, pages 3853-3865, see entire document.	1-11
Y	US 5,723,585 A (BAKER et al) 03 March 1998 (03.03.1998), column 4, lines 9-35 and column 8, line 66-column 9, line 23.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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08 May 2000 (08.05.2000)	06 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Patricia Robinson for Dorothea Laurence Christopher Low Telephone No. 703-308-0196

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Continuation of B. FIELDS SEARCHED Item 3: STN, BIOSIS, CAPLUS, MEDLINE
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(72) Inventors; and

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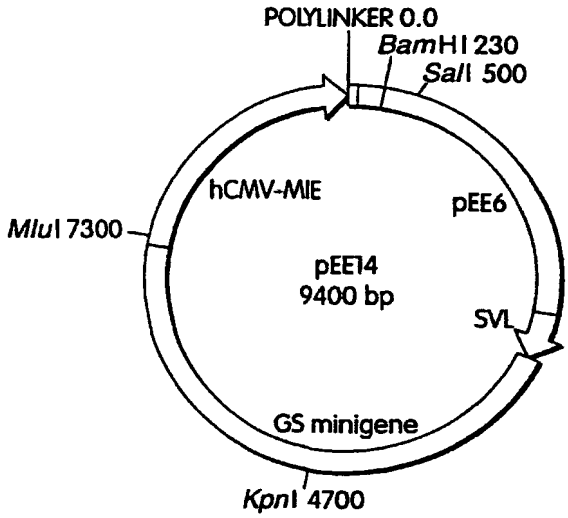

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(57) Abstract

The invention features a nucleic acid, vector, cells, and processes for expressing recombinant human alpha–fetoprotein (rHuAFP) in cultured mammalian cells.

*(Referred to in PCT Gazette No. 45/2000, Section II)

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VERSION

INTERNATIONAL SEARCH REPORT

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Patricia Robinson
Christopher Low

Telephone No. 703-308-0196

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